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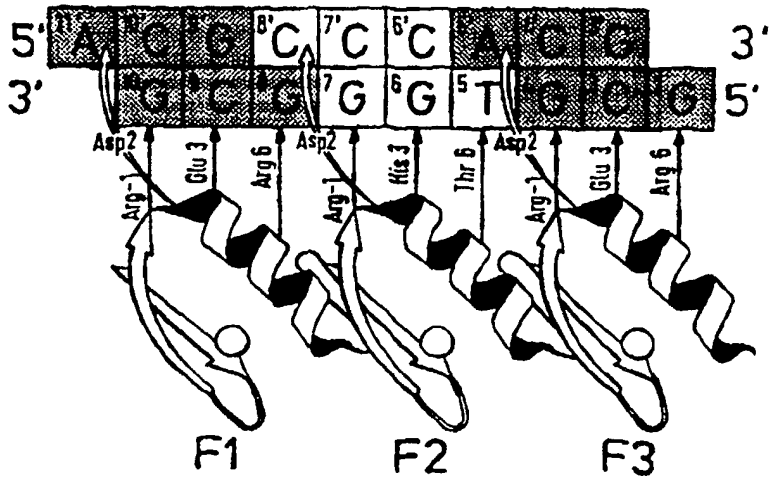
(54) **Title:** NUCLEIC ACID BINDING PROTEINS

(57) **Abstract**

The invention provides a method for preparing a nucleic acid binding protein of the Cys2–His2 zinc finger class capable of binding to a nucleic acid quadruplet in a target nucleic acid sequence, wherein binding to base 4 of the quadruplet by an α–helical zinc finger nucleic acid binding motif in the protein is determined as follows: if base 4 in the quadruplet is A, then position +6 in the α–helix is Gln and position ++2 is not Asp; and if base 4 in the quadruplet is C, then position +6 in the α–helix may be any residue, as long as position ++2 in the α–helix is not Asp.



Nucleic Acid Binding Proteins

The present invention relates to nucleic acid binding proteins. In particular, the invention relates to a method for designing a protein which is capable of binding to any predefined nucleic acid sequence.

Protein-nucleic acid recognition is a commonplace phenomenon which is central to a large number of biomolecular control mechanisms which regulate the functioning of eukaryotic and prokaryotic cells. For instance, protein-DNA interactions form the basis of the regulation of gene expression and are thus one of the subjects most widely studied by molecular biologists.

A wealth of biochemical and structural information explains the details of protein-DNA recognition in numerous instances, to the extent that general principles of recognition have emerged. Many DNA-binding proteins contain independently folded domains for the recognition of DNA, and these domains in turn belong to a large number of structural families, such as the leucine zipper, the "helix-turn-helix" and zinc finger families.

Despite the great variety of structural domains, the specificity of the interactions observed to date between protein and DNA most often derives from the complementarity of the surfaces of a protein α-helix and the major groove of DNA [Klug, (1993) Gene 135:83-92]. In light of the recurring physical interaction of α-helix and major groove, the tantalising possibility arises that the contacts between particular amino acids and DNA bases could be described by a simple set of rules; in effect a stereochemical recognition code which relates protein primary structure to binding-site sequence preference.

It is clear, however, that no code will be found which can describe DNA recognition by all DNA-binding proteins. The structures of numerous complexes show significant differences in the way that the recognition α-helices of DNA-binding proteins from different structural families interact with the major groove of DNA, thus precluding similarities in patterns of recognition. The majority of known DNA-binding motifs are not particularly versatile, and

elucidated for the interactions of classical zinc fingers with nucleic acid. In this case a pattern of rules is provided which covers binding to all nucleic acid sequences.

According to a first aspect of the present invention, therefore, we provide a method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid quadruplet in a target nucleic acid sequence, wherein binding to base 4 of the quadruplet by an α-helical zinc finger nucleic acid binding motif in the protein is determined as follows:

a) if base 4 in the quadruplet is A, then position +6 in the α-helix is Gln and + +2 is not Asp;

b) if base 4 in the quadruplet is C, then position +6 in the α-helix may be any residue, as long as position + +2 in the α-helix is not Asp.

Preferably, binding to base 4 of the quadruplet by an α-helical zinc finger nucleic acid binding motif in the protein is additionally determined as follows:

c) if base 4 in the quadruplet is G, then position +6 in the α-helix is Arg; or position +6 is Ser or Thr and position + +2 is Asp;

d) if base 4 in the quadruplet is T, then position +6 in the α-helix is Ser or Thr and position + +2 is Asp.

The quadruplets specified in the present invention are overlapping, such that, when read 3' to 5' on the -strand of the nucleic acid, base 4 of the first quadruplet is base 1 of the second, and so on. Accordingly, in the present application, the bases of each quadruplet are referred by number, from 1 to 4, 1 being the 3' base and 4 being the 5' base.

All of the nucleic acid-binding residue positions of zinc fingers, as referred to herein, are numbered from the first residue in the α-helix of the finger, ranging from +1 to +9. "-1" refers to the residue in the framework structure immediately preceding the α-helix in a Cys2-His2 zinc finger polypeptide.

acid, since it is this strand which is aligned 3' to 5'. These conventions are followed in the nomenclature used herein. It should be noted, however, that in nature certain fingers, such as finger 4 of the protein GLI, bind to the + strand of nucleic acid: see Suzuki *et al.*, (1994) NAR 22:3397-3405 and Pavletich and Pabo, (1993) Science 261:1701-1707. The incorporation of such fingers into nucleic acid binding molecules according to the invention is envisaged.

The invention provides a solution to a problem hitherto unaddressed in the art, by permitting the rational design of polypeptides which will bind nucleic acid quadruplets whose 5' residue is other than G. In particular, the invention provides for the first time a solution for the design of polypeptides for binding quadruplets containing 5' A or C.

Position +6 in the α-helix is generally responsible for the interaction with the base 4 of a given quadruplet in the target. According to the present invention, an A at base 4 interacts with a Glutamine (Gln or Q) at position +6, while a C at base 4 will interact with any amino acid provided that position ++2 is not Aspartic acid (Asp or D).

The present invention concerns a method for preparing nucleic acid binding proteins which are capable of binding nucleic acid. Thus, whilst the solutions provided by the invention will result in a functional nucleic acid binding molecule, it is possible that naturally-occurring zinc finger nucleic acid binding molecules may not follow some or all of the rules provided herein. This does not matter, because the aim of the invention is to permit the design of the nucleic acid binding molecules on the basis of nucleic acid sequence, and not the converse. This is why the rules, in certain instances, provide for a number of possibilities for any given residue. In other instances, alternative residues to those given may be possible. The present invention, thus, does not seek to provide every solution for the design of a binding protein for a given target nucleic acid. It does, however, provide for the first time a complete solution allowing a functional nucleic acid binding protein to be constructed for any given nucleic acid quadruplet.

position +2 in the helix is responsbile for determining the binding to base 1 of the quadruplet. In doing so, it cooperates synergistically with position +6, which determines binding at base 4 in the quadruplet. bases 1 and 4 being overlapping in adjacent quadruplets.

A zinc finger binding motif is a structure well known to those in the art and defined in, for example, Miller *et al.*, (1985) EMBO J. 4:1609-1614; Berg (1988) PNAS (USA) 85:99-102; Lee *et al.*, (1989) Science 245:635-637; see International patent applications WO 96/06166 and WO 96/32475, corresponding to USSN 08/422,107, incorporated herein by reference.

As used herein, "nucleic acid" refers to both RNA and DNA, constructed from natural nucleic acid bases or synthetic bases, or mixtures thereof. Preferably, however, the binding proteins of the invention are DNA binding proteins.

In general, a preferred zinc finger framework has the structure:

(A) $X_{0-2}\ C\ X_{1-5}\ C\ X_{9-14}\ H\ X_{3-5}\ {}^{H}/_{C}$

where X is any amino acid, and the numbers in subscript indicate the possible numbers of residues represented by X.

In a preferred aspect of the present invention, zinc finger nucleic acid binding motifs may be represented as motifs having the following primary structure:

(B) $X^a\ C\ X_{2-4}\ C\ X_{2-3}\ F\ X^c\ X\ X\ X\ X\ L\ X\ X\ H\ X\ X\ X^b\ H$ - linker

-1 1 2 3 4 5 6 7 8 9

wherein X (including X^a, X^b and X^c) is any amino acid. X_{2-4} and X_{2-3} refer to the presence of 2 or 4, or 2 or 3, amino acids, respectively. The Cys and His residues, which together

Preferably, the linker is T-G-E-K or T-G-E-K-P.

As set out above, the major binding interactions occur with amino acids -1, +2, +3 and +6. Amino acids +4 and +7 are largely invariant. The remaining amino acids may be essentially any amino acids. Preferably, position +9 is occupied by Arg or Lys. Advantageously, positions +1, +5 and +8 are not hydrophobic amino acids, that is to say are not Phe, Trp or Tyr.

In a most preferred aspect, therefore, bringing together the above, the invention allows the definition of every residue in a zinc finger nucleic acid binding motif which will bind specifically to a given nucleic acid quadruplet.

The code provided by the present invention is not entirely rigid; certain choices are provided. For example, positions +1, +5 and +8 may have any amino acid allocation, whilst other positions may have certain options: for example, the present rules provide that, for binding to a central T residue, any one of Ala, Ser or Val may be used at +3. In its broadest sense, therefore, the present invention provides a very large number of proteins which are capable of binding to every defined target nucleic acid quadruplet.

Preferably, however, the number of possibilities may be significantly reduced. For example, the non-critical residues +1, +5 and +8 may be occupied by the residues Lys, Thr and Gln respectively as a default option. In the case of the other choices, for example, the first-given option may be employed as a default. Thus, the code according to the present invention allows the design of a single, defined polypeptide (a "default" polypeptide) which will bind to its target quadruplet.

In a further aspect of the present invention, there is provided a method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a target nucleic acid sequence, comprising the steps of:

residues known to affect binding to bases at which the natural and desired targets differ. Otherwise, mutation of the model fingers should be concentrated upon residues -1, +2, +3 and +6 as provided for in the foregoing rules.

In order to produce a binding protein having improved binding, moreover, the rules provided by the present invention may be supplemented by physical or virtual modelling of the protein/nucleic acid interface in order to assist in residue selection.

Zinc finger binding motifs designed according to the invention may be combined into nucleic acid binding proteins having a multiplicity of zinc fingers. Preferably, the proteins have at least two zinc fingers. In nature, zinc finger binding proteins commonly have at least three zinc fingers, although two-zinc finger proteins such as Tramtrack are known. The presence of at least three zinc fingers is preferred. Binding proteins may be constructed by joining the required fingers end to end, N-terminus to C-terminus. Preferably, this is effected by joining together the relevant nucleic acid coding sequences encoding the zinc fingers to produce a composite coding sequence encoding the entire binding protein. The invention therefore provides a method for producing a nucleic acid binding protein as defined above, wherein the nucleic acid binding protein is constructed by recombinant DNA technology, the method comprising the steps of:

a) preparing a nucleic acid coding sequence encoding two or more zinc finger binding motifs as defined above, placed N-terminus to C-terminus;
b) inserting the nucleic acid sequence into a suitable expression vector; and
c) expressing the nucleic acid sequence in a host organism in order to obtain the nucleic acid binding protein.

A "leader" peptide may be added to the N-terminal finger. Preferably, the leader peptide is MAEEKP.

The nucleic acid encoding the nucleic acid binding protein according to the invention can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid)

can be amplified by PCR and be directly transfected into the host cells without any replication component.

Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript® vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both *E. coli* replication origin and *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up nucleic acid binding protein nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to amplification

UV5 promoter. This system has been employed successfully for over-production of many proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int- phage such as the CE6 phage which is commercially available (Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL) , vectors containing the trc promoters such as pTrcHisXpressTm (Invitrogen) or pTrc99 (Pharmacia Biotech, SE) or vectors containing the tac promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (New England Biolabs, MA, USA).

Moreover, the nucleic acid binding protein gene according to the invention preferably includes a secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it will be produced as a soluble native peptide rather than in an inclusion body. The peptide may be recovered from the bacterial periplasmic space, or the culture medium, as appropriate.

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a Saccharomyces cerevisiae gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the a- or α-factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phospho glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PHO5 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the

contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding nucleic acid binding protein.

An expression vector includes any vector capable of expressing nucleic acid binding protein nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of expression of such DNAs. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector, that upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those with ordinary skill in the art and include those that are replicable in eukaryotic and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. For example, DNAs encoding nucleic acid binding protein may be inserted into a vector suitable for expression of cDNAs in mammalian cells, e.g. a CMV enhancer-based vector such as pEVRF (Matthias, et al., (1989) NAR 17, 6418).

Particularly useful for practising the present invention are expression vectors that provide for the transient expression of DNA encoding nucleic acid binding protein in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high levels of nucleic acid binding protein. For the purposes of the present invention, transient expression systems are useful e.g. for identifying nucleic acid binding protein mutants, to identify potential phosphorylation sites, or to characterise functional domains of the protein.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing nucleic acid binding protein expression and function are known to those skilled in the art. Gene presence, amplification and/or

binding protein may be empirically determined and optimised for a particular cell and assay.

Host cells are transfected or, preferably, transformed with the above-captioned expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Heterologous DNA may be introduced into host cells by any method known in the art, such as transfection with a vector encoding a heterologous DNA by the calcium phosphate coprecipitation technique or by electroporation. Numerous methods of transfection are known to the skilled worker in the field. Successful transfection is generally recognised when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press).

Transfected or transformed cells are cultured using media and culturing methods known in the art, preferably under conditions, whereby the nucleic acid binding protein encoded by the DNA is expressed. The composition of suitable media is known to those in the art, so that they can be readily prepared. Suitable culturing media are also commercially available.

In a further aspect, the invention also provides means by which the binding of the protein designed according to the rules can be improved by randomising the proteins and selecting for improved binding. In this aspect, the present invention represents an improvement of the method set forth in WO 96/06166. Thus, zinc finger molecules designed according to the invention may be subjected to limited randomisation and subsequent selection, such as by phage display, in order to optimise the binding characteristics of the molecule.

affinity purification. The phage are then amplified by passage through a bacterial host, and subjected to further rounds of selection and amplification in order to enrich the mutant pool for the desired phage and eventually isolate the preferred clone(s). Detailed methodology for phage display is known in the art and set forth, for example, in US Patent 5,223,409; Choo and Klug, (1995) Current Opinions in Biotechnology 6:431-436; Smith. (1985) Science 228:1315-1317; and McCafferty *et al.*, (1990) Nature 348:552-554; all incorporated herein by reference. Vector systems and kits for phage display are available commercially, for example from Pharmacia.

Randomisation of the zinc finger binding motifs produced according to the invention is preferably directed to those residues where the code provided herein gives a choice of residues. For example, therefore, positions +1, +5 and +8 are advantageously randomised, whilst preferably avoiding hydrophobic amino acids; positions involved in binding to the nucleic acid, notably -1, +2, +3 and +6, may be randomised also, preferably within the choices provided by the rules of the present invention.

Preferably, therefore, the "default" protein produced according to the rules provided by the invention can be improved by subjecting the protein to one or more rounds of randomisation and selection within the specified parameters.

nucleic acid binding proteins according to the invention may be employed in a wide variety of applications, including diagnostics and as research tools. Advantageously, they may be employed as diagnostic tools for identifying the presence of nucleic acid molecules in a complex mixture. nucleic acid binding molecules according to the invention can differentiate single base pair changes in target nucleic acid molecules.

Accordingly, the invention provides a method for determining the presence of a target nucleic acid molecule, comprising the steps of:

a) preparing a nucleic acid binding protein by the method set forth above which is specific for the target nucleic acid molecule;

nucleic acid cleaving domain is fused to a nucleic acid binding domain comprising a zinc finger as described herein.

The invention is described below, for the purpose of illustration only, in the following examples, with reference to the figures, in which:

Figure 1 illustrates the design of a zinc finger binding protein specific for a G12V mutant ras oncogene;

Figure 2 illustrates the binding specificity of the binding protein for the oncogene as opposed to the wild-type ras sequence; and

Figure 3 illustrates the results of an ELISA assay performed using the anti-ras binding protein with both wild-type and mutant target nucleic acid sequences;

Figure 4 illustrates interactions between the Zif268 DNA-binding domain and DNA. (a) Schematic diagram of modular recognition between the three zinc fingers of Zif268 and triplet subsites of an optimised DNA binding site. Straight arrows indicate the stereochemical juxtapostioning of recognition residues with bases of the contacted G-rich DNA strand. Note that since the N-terminal finger contacts the 3' end of the DNA and the C-terminal finger the 5' end, binding to the G-rich strand is said to be antiparallel. (b) View of Zif268 finger 3 bound to DNA, showing the possibility of interaction with both DNA strands. Co-ordinates from Pavletich & Pabo, (1991) Science 252:809-817. (c) The potential hydrogen bonding network between bases on both strands of the DNA and positions -1 (Arg) and 2 (Asp) of finger 3 (Pavletich & Pabo 1991). (d) Schematic diagram of recognition between the three zinc fingers of Zif268 and an optimised DNA binding site including 'cross-strand' interactions. Recognition contacts between Asp2 of each finger and the parallel DNA strand (shown by curly arrows) mean that each finger binds overlapping, 4 bp subsites;

Example 1

Construction of a zinc finger protein

The target selected for the zinc finger nucleic acid binding protein is the activating point mutation of the human EJ bladder carcinoma *ras* oncogene, which was the first DNA lesion reported to confer transforming properties on a cellular proto-oncogene. Since the original discovery, *ras* gene mutations have been found to occur at high frequencies in a variety of human cancers and are established targets for the diagnosis of oncogenesis at early stages of tumour growth.

The EJ bladder carcinoma mutation is a single nucleotide change in codon 12 of H-*ras*, which results in a mutation from GGC to GTC at this position. A zinc finger peptide is designed to bind a 10bp DNA site assigned in the noncoding strand of the mutant *ras* gene, such that three fingers contact 'anticodons' 10, 11 and 12 in series, as shown in Fig. 1, plus the 5' preceding G (on the +strand of the DNA). The rationale of this assignment takes into account the fact that zinc fingers make most contacts to one DNA strand, and the mutant noncoding strand carries an adenine which can be strongly discriminated from the cytosine present in the wild-type ras, by a bidentate contact from an asparagine residue.

The first finger of the designer lead peptide is designed according to the rules set forth herein starting from a Zif268 finger 2 model to bind the quadruplet 5'-GCCG-3', which corresponds to 'anticodon' 10 of the designated binding site plus one 3' base. The finger has the following sequence:

F Q C R I C M R N F S D R S S L T R H T R T H T G E K P

-1 1 2 3 4 5 6 7 8 9

A DNA coding sequence encoding this polypeptide is constructed from synthesised oligonucleotides.

According to the recognition rules, the first finger of the lead peptide could contact cytosine using one of Asp, Glu, Ser or Thr in the third α-helix position. To determine the optimal contact, the codon for helical position 3 of finger 1 is engineered by cassette mutagenesis to have position 1= A/G, position 2=A/C/G and position 3=C/G. Therefore in addition to Asp, Glu, Ser and Thr, the randomisation also specifies Ala, Arg, Asn, Gly and Lys. Selections from this mini-library are over one round of phage binding to 5nM mutant DNA oligo in 100 µl PBS containing 50µM $ZnCl_2$, 2% (w/v) fat-free dried milk (Marvel) and 1% (v/v) Tween-20, with 1µg poly dIdC as competitor, followed by six washes with PBS containing 50µM $ZnCl_2$ and 1% (v/v) Tween-20. Bound phage are eluted with 0.1M triethylamine for 3 mins, and immediately transferred to an equal volume of 1M Tris-Cl pH 7.4.

A single round of randomisation and selection is found to be sufficient to improve the affinity of the lead zinc finger peptide to this standard. A small library of mutants is constructed with limited variations specifically in the third α-helical position (+3) of finger 1 of the designed peptide. Selection from this library yields an optimised DNA-binding domain with asparagine at the variable position, which is able to bind the mutant *ras* sequence with an apparent K_d of 3nM, i.e. equal to that of the wild-type Zif268 DNA-binding domain (Fig. 2). The selection of asparagine at this position to bind opposite a cytosine is an unexpected deviation from the recognition rules, which normally pair asparagine with adenine.

The selection of asparagine is, however, consistent with physical considerations of the protein-DNA interface. In addition to the classical bidentate interaction of asparagine and adenine observed in zinc finger-DNA complexes, asparagine has been observed to bridge a base-pair step in the major groove of DNA, for example in the co-crystal structures of the GCN4 DNA-binding domain. A number of different base-pair steps provide the correct stereochemical pairings of hydrogen bond donors and acceptors which could satisfy asparagine, including the underlined step G<u>CC</u> of ras 'anticodon' 10. Although asparagine in position 3 of the zinc finger helix would not normally be positioned to bridge a base-pair

removed by washing the beads 6 times with PBS containing 50μM $ZnCl_2$ and 1% (v/v) Tween-20. The beads are subsequently incubated for 1h at RT with anti-M13 IgG conjugated to horseradish peroxidase (Pharmacia Biotech) diluted 1:5000 in PBS containing 50μM $ZnCl_2$ and 2% (w/v) fat-free dried milk (Marvel). Excess antibody is removed by washing 6 times with PBS containing 50μM $ZnCl_2$ and 0.05% (v/v) Tween, and 3 times with PBS containing 50μM $ZnCl_2$. The ELISA is developed with 0.1mg/ml tetramethylbenzidine (Sigma) in 0.1M sodium acetate pH5.4 containing 2μl of fresh 30% hydrogen peroxide per 10ml buffer, and after approximately 1 min, stopped with an equal volume of 2M H_2SO_4. The reaction produces a yellow colour which is quantitated by subtracting the absorbance at 650nm from the absorbance at 450nm. It should be noted that in this protocol the ELISA is not made competitive, however, soluble (non biotinylated) wild-type *ras* DNA could be included in the binding reactions, possibly leading to higher discrimination between wild-type and mutant *ras*.

Phage are retained specifically by DNA bearing the mutant, but not the wild-type *ras* sequence, allowing the detection of the point mutation by ELISA (Fig. 3).

Example 4

Design of an anti-HIV zinc finger

The sequence of the HIV TAR, the region of the LTR which is responsible for trans-activation by Tat, is known (Jones and Peterlin, (1994) Ann. Rev. Biochem. 63:717-743). A sequence with the TAT region is identified and a zinc finger polypeptide designed to bind thereto.

The selected sequence is 5' - AGA GAG CTC - 3', which is the complement of nucleotides +34 to +42 of HIV. The corresponding amino acids required in fingers 1, 2 and 3 of a zinc finger binding protein are determined according to the rules set forth above, as follows:

vector. Electrocompetent TG1 cells are transformed with the recombinant vector. Single colonies of tranformants are grown overnight in 2xTY containing 50μM $ZnCl_2$ 15μg/ml tetracycline. Single stranded DNA is prepared from phage in the culture supernatant and sequenced with Sequenase 2.0 (United States Biochemical).

The polypeptide designed according to the invention is then tested for binding to HIV DNA and positive results are obtained.

Example 5

Alanine mutagenesis of the Asp2 in finger 3 is carried out on the wild-type Zif268 DNA-binding domain and four related peptides isolated from the phage display library as follows (see also Fig. 5):

E. coli TG1 cells are tranfected with fd phage displaying zinc fingers. Colony PCR is performed with one primer containing a single mismatch to create the Asp to Ala change in finger 3. Cloning of PCR product in phage vector is as described previously (Choo, Y. & Klug, A. (1994) Proc. Natl. Acad. Sci. USA 91, 11163-11167; Choo, Y. & Klug, A. (1994) Proc. Natl. Acad. Sci. USA 91, 11168-11172). Briefly, forward and backward PCR primers contained unique restriction sites for *Not* I or *Sfi* I respectively and amplified an approximately 300 base pair region encompassing three zinc fingers. PCR products are digested with *Sfi* I and *Not* I to create cohesive ends and are ligated to 100ng of similarly digested fd-Tet-SN vector. Electrocompetent TG1 cells are transformed with the recombinant vector. Single colonies of tranformants are grown overnight in 2xTY containing 50μM $ZnCl_2$ 15μg/ml tetracycline. Single stranded DNA is prepared from phage in the culture supernatant and sequenced with Sequenase 2.0 (United States Biochemical).

The peptides are chosen for this experiment on the basis of the identity of the residue at position 6 of the middle finger. Peptide F2-Arg, which contains Arg at position 6 of finger 2, is chosen since it should specify 5'-G in the 'middle' cognate triplet regardless of the

2. As would be expected, according to the hypothesis set out in the introduction, the mutation affects binding at the 5' position, while the specificity at the middle and 3' position remains unchanged.

The mutation generally leads to a broadening of specificity, for instance in Zif268 where removal of Asp2 in finger 3 results in a protein which is unable to discriminate the 5' base of the middle triplet (Fig. 6a). However, the expectation that a new 5' base-specificity for the mutants might correlate to the identity of position 6 in finger 2, is not borne out. For example F2-Gly would be expected to lose sequence discrimination but, although specificity is adversely affected, a slight preference for T is discernible (Fig. 6b). Similarly, F2-Val and F2-Asn which might have been expected to acquire specificity for one nucleotide, instead have their specificities altered by the mutation (Fig. 6c, d) - the F2-Val mutant allows G, A and T but not C, and the F2-Asn mutant appears to discriminate against both pyrimidines. In the absence of a larger database it is not possible to deduce whether these apparent specificities are the result of amino acid-base contacts from position 6 of finger 2, and if so whether these are general interactions which should be regarded as recognition rules. The apparent discrimination of F2-Gly in particular, suggests that this is unlikely to be the case, but rather that in these particular examples, other mechanisms are involved in determining sequence bias.

In contrast to the loss of discrimination seen for the other four peptides, F2-Arg continues to specify guanine in the 5' position of the middle triplet regardless of the mutation in finger 3 (fig 3e). In this case, the specificity is derived from the strong interaction between guanine and Arg6 in finger 2. This contact has been observed a number of times in zinc finger co-crystal structures (Pavletich, N. P. & Pabo, C. O. (1993) Science 261, 1701-1707; Fairall, L., Schwabe, J. W. R., Chapman, L., Finch, J. T. & Rhodes, D. (1993) Nature (London) 366, 483-487; Fairall, L., Schwabe, J. W. R., Chapman, L., Finch, J. T. & Rhodes, D. (1993) Nature (London) 366, 483-487; Kim, C. & Berg, J. M. (1996) Nature Str. Biol. 3, 940-945) and is the only recognition rule which relates amino acid identity at position 6 to a nucleotide preference at the 5' position of a cognate triplet (Choo, Y.& Klug, A. (1997) Curr. Opin. Str. Biol. 7, 117-125). This interaction is compatible

To determine the contribution of Asp2 in finger 3 to the binding strength, apparent equilibrium dissociation constants are determined for Zif268 and F2-Arg before and after the Ala mutation (Fig. 7). Procedures are as described previously (Choo and Klug, 1994). Briefly, appropriate concentrations of 5'-biotinylated DNA binding sites are added to equal volumes of phage solution described above. Binding is allowed to proceed for one hour at 20°C. DNA is captured with streptavidin-coated paramagnetic beads (500μg/well). The beads are washed 6 times with PBS/Zn containing 1% Tween, then 3 times with PBS/Zn. Bound phage are detected by ELISA with horseradish peroxidase-conjugated anti-M13 IgG (Pharmacia Biotech) and quantitated using SOFTMAX 2.32 (Molecular Devices). Binding data are plotted and analysed using Kaleidagraph (Abelbeck Software).

Both mutants show approximately a four-fold reduction in affinity for their respective binding sites under the conditions used. The reduction is likely a direct result of abolishing contacts from Asp2, rather than a consequence of changes in binding specificity at the 5' position of the middle triplet, since the mutant Zif268 loses all specificity while F2-Arg registers no change in specificity. However, note that two stabilising interactions are abolished: an intramolecular buttressing interaction with Arg-1 on finger 3 and also the intermolecular contact with the secondary DNA strand. An independent comparison of wild-type Zif268 binding to its consensus binding site flanked by G/T or A/C also found a five-fold reduction in affinity for those sites which are unable to satisfy a contact from Asp2 to the secondary DNA strand (Smirnoff, A. H. & Milbrandt, J. (1995) Mol. Cel. Biol. 15, 2275-2287). While the effects of perturbations in the DNA structure cannot be discounted in this case, the results of both experiments would seem to suggest that the reduction in binding affinity results from loss of the protein-DNA contact. Nevertheless, the intramolecular contact between positions -1 and 2 in a zinc finger, is a further level of synergy which may have to be taken into account before the full picture emerges, describing the possible networks of contacts which occur at the protein-DNA interface in the region of the overlapping subsites.

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..264

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GCA GAA GAG AAG CCT TTT CAG TGT CGA ATC TGC ATG CGT AAC TTC AGC        48
Ala Glu Glu Lys Pro Phe Gln Cys Arg Ile Cys Met Arg Asn Phe Ser
 1               5                  10                  15

GAT CGT AGT AGT CTT ACC CGC CAC ACG AGG ACC CAC ACA GGC GAG AAG        96
Asp Arg Ser Ser Leu Thr Arg His Thr Arg Thr His Thr Gly Glu Lys
            20                  25                  30

CCT TTT CAG TGT CGA ATC TGC ATG CGT AAC TTC AGC AGG AGC GAT AAC       144
Pro Phe Gln Cys Arg Ile Cys Met Arg Asn Phe Ser Arg Ser Asp Asn
        35                  40                  45

CTT ACG AGA CAC CTA AGG ACC CAC ACA GGC GAG AAG CCT TTT CAG TGT       192
Leu Thr Arg His Leu Arg Thr His Thr Gly Glu Lys Pro Phe Gln Cys
    50                  55                  60

CGA ATC TGC ATG CGT AAC TTC AGG CAA GCT GAT CAT CTT CAA GAG CAC       240
Arg Ile Cys Met Arg Asn Phe Arg Gln Ala Asp His Leu Gln Glu His
65                  70                  75                  80

CTA AAG ACC CAC ACA GGC GAG AAG                                      264
Leu Lys Thr His Thr Gly Glu Lys
                85
```

Claims:

1. A method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid quadruplet in a target nucleic acid sequence, wherein binding to base 4 of the quadruplet by an α-helical zinc finger nucleic acid binding motif in the protein is determined as follows:

a) if base 4 in the quadruplet is A, then position +6 in the α-helix is Gln and position ++2 is not Asp;

b) if base 4 in the quadruplet is C, then position −6 in the α-helix may be any residue, as long as position ++2 in the α-helix is not Asp.

2. A method according to claim 1, wherein binding to base 4 of the quadruplet by an α-helical zinc finger nucleic acid binding motif in the protein is additionally determined as follows:

c) if base 4 in the quadruplet is G, then position +6 in the α-helix is Arg; or position +6 is Ser or Thr and position ++2 is Asp;

d) if base 4 in the quadruplet is T, then position +6 in the α-helix is Ser or Thr and position ++2 is Asp.

3. A method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid quadruplet in a target nucleic acid sequence, wherein binding to each base of the quadruplet by an α-helical zinc finger nucleic acid binding motif in the protein is determined as follows:

a) if base 4 in the quadruplet is G, then position +6 in the α-helix is Arg; or position +6 is Ser or Thr and position ++2 is Asp;

b) if base 4 in the quadruplet is A, then position +6 in the α-helix is Gln and position ++2 is not Asp;

7. A method according to any one of claims 4 to 6 wherein X^b is T or I.

8. A method according to any one of claims 4 to 7 wherein X_{2-3} is G-K-A, G-K-C, G-K-S, G-K-G, M-R-N or M-R.

9. A method according to any one of claims 4 to 8 wherein the linker is T-G-E-K or T-G-E-K-P.

10. A method according to any one of claims 4 to 9 wherein position +9 is R or K.

11. A method according to any one of claims 4 to 10 wherein positions +1, +5 and +8 are not occupied by any one of the hydrophobic amino acids, F, W or Y.

12. A method according to claim 11 wherein positions +1, +5 and +8 are occupied by the residues K, T and Q respectively.

13. A method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a target nucleic acid sequence, comprising the steps of:

a) selecting a model zinc finger domain from the group consisting of naturally occurring zinc fingers and consensus zinc fingers; and

b) mutating the finger according to the rules set in any one of claims 1 to 3.

14. A method according to claim 13, wherein the model zinc finger is a consensus zinc finger whose structure is selected from the group consisting of the consensus structure P Y K C P E C G K S F S Q K S D L V K H Q R T H T G, and the consensus structure P Y K C S E C G K A F S Q K S N L T R H Q R I H T G E K P.

22. A method according to claim 21, comprising the steps of:

a) preparing a nucleic acid construct capable of expressing a fusion protein comprising the nucleic acid binding protein and a minor coat protein of a filamentous bacteriophage;
b) preparing further nucleic acid constructs capable of expressing a fusion protein comprising a selectively mutated nucleic acid binding protein and a minor coat protein of a filamentous bacteriophage;
c) causing the fusion proteins defined in steps (a) and (b) to be expressed on the surface of bacteriophage transformed with the nucleic acid constructs;
d) assaying the ability of the bacteriophage to bind the target nucleic acid sequence and selecting the bacteriophage demonstrating superior binding characteristics.

23. A method according to any one of claims 20 to 22 wherein the nucleic acid binding protein is selectively randomised at any one of positions +1, +5, +8, -1, +2, +3 or +6.

24. A method for determining the presence of a target nucleic acid molecule, comprising the steps of:

a) preparing a nucleic acid binding protein by the method of any preceding claim which is specific for the target nucleic acid molecule;
b) exposing a test system comprising the target nucleic acid molecule to the nucleic acid binding protein under conditions which promote binding, and removing any nucleic acid binding protein which remains unbound;
c) detecting the presence of the nucleic acid binding protein in the test system.

25. A method according to claim 24, wherein the presence of the nucleic acid binding protein in the test system is detected by means of an antibody.

26. A method according to claim 24 or claim 25 wherein the nucleic acid binding protein, in use, is displayed on the surface of a filamentous bacteriophage and the presence

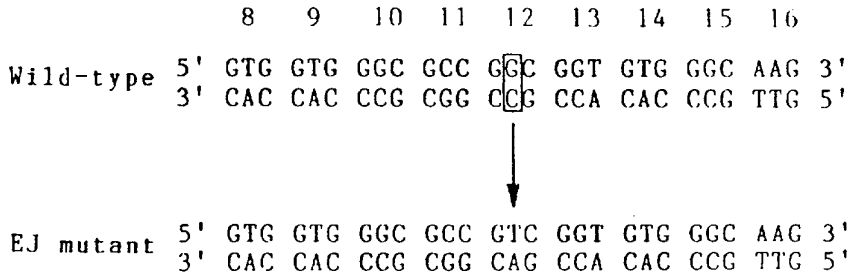


FIG. 1A

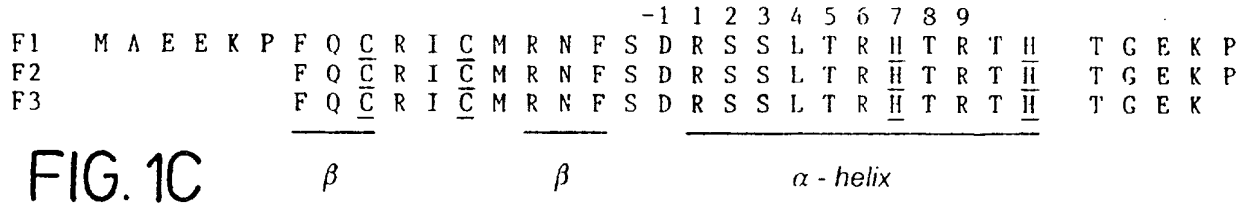
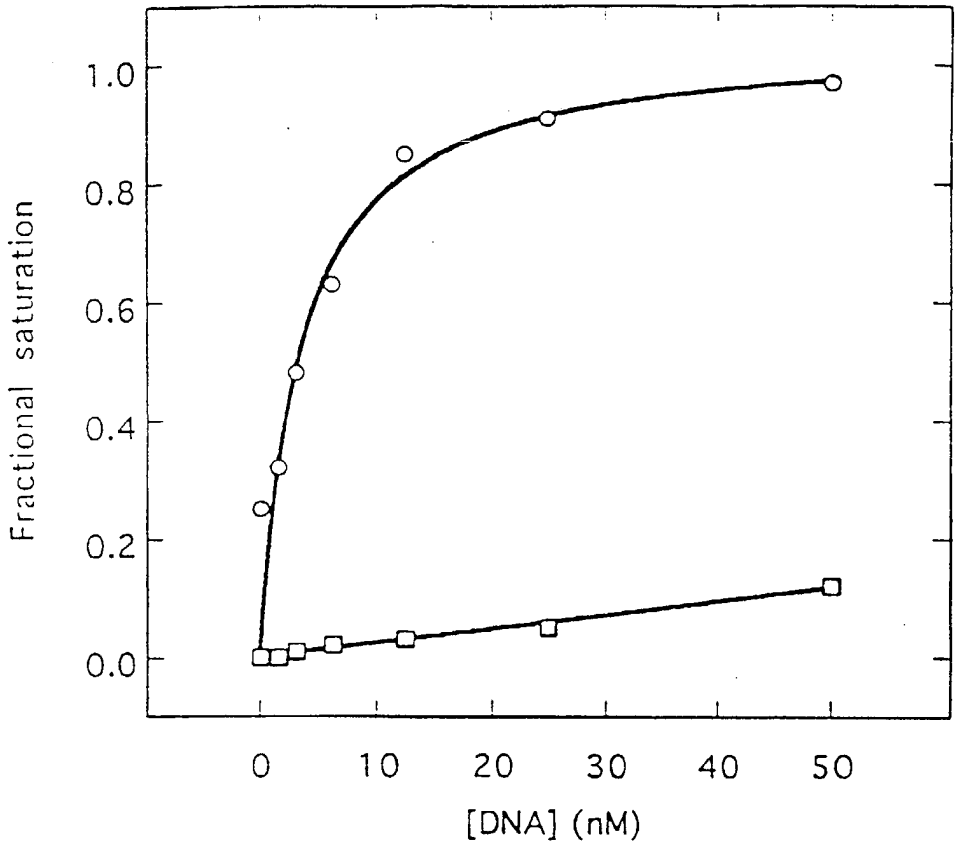


FIG. 1C


Fractional saturation
1.0
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0.6
0.4
0.2
0.0
0
10
20
30
40
50
[DNA] (nM)

FIG. 2

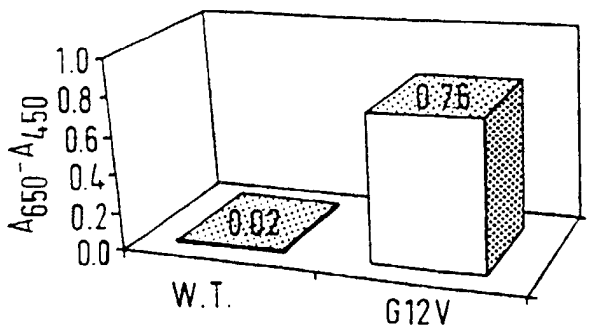
$A_{650}-A_{450}$
1.0
0.8
0.6
0.4
0.2
0.0
0.02
0.76
W.T.
G12V

FIG. 3

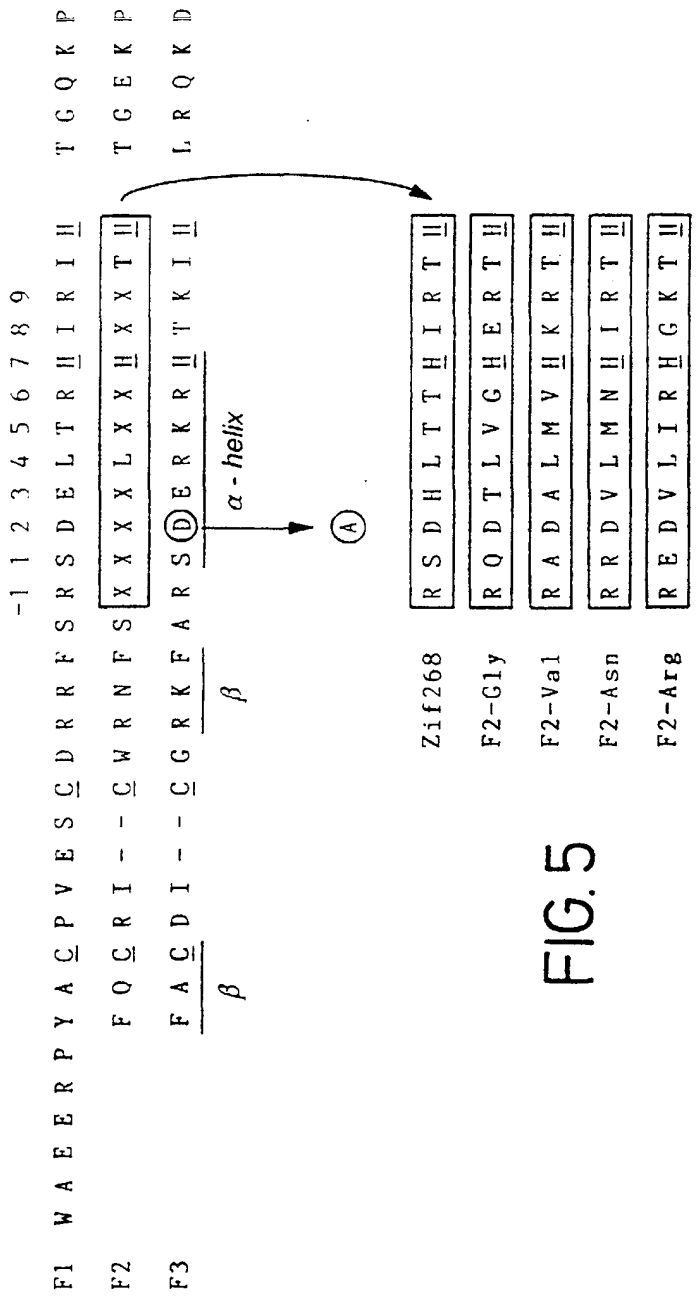
-1 1 2 3 4 5 6 7 8 9
F1 W A E E R P Y A C P V E S C D R R F S R S D E L T R H I R I H T G Q K P
F2 F Q C R I - - C W R N F S X X X L X X H X X T H T G E K P
F3 F A C D I - - C G R K F A R S D E R K R H T K I H L R Q K D
β
β
α - helix
A
Zif268 R S D H L T T H I R T H
F2-Gly R Q D T L V G H E R T H
F2-Val R A D A L M V H K R T H
F2-Asn R R D V L M N H I R T H
F2-Arg R E D V L I R H G K T H

FIG. 5

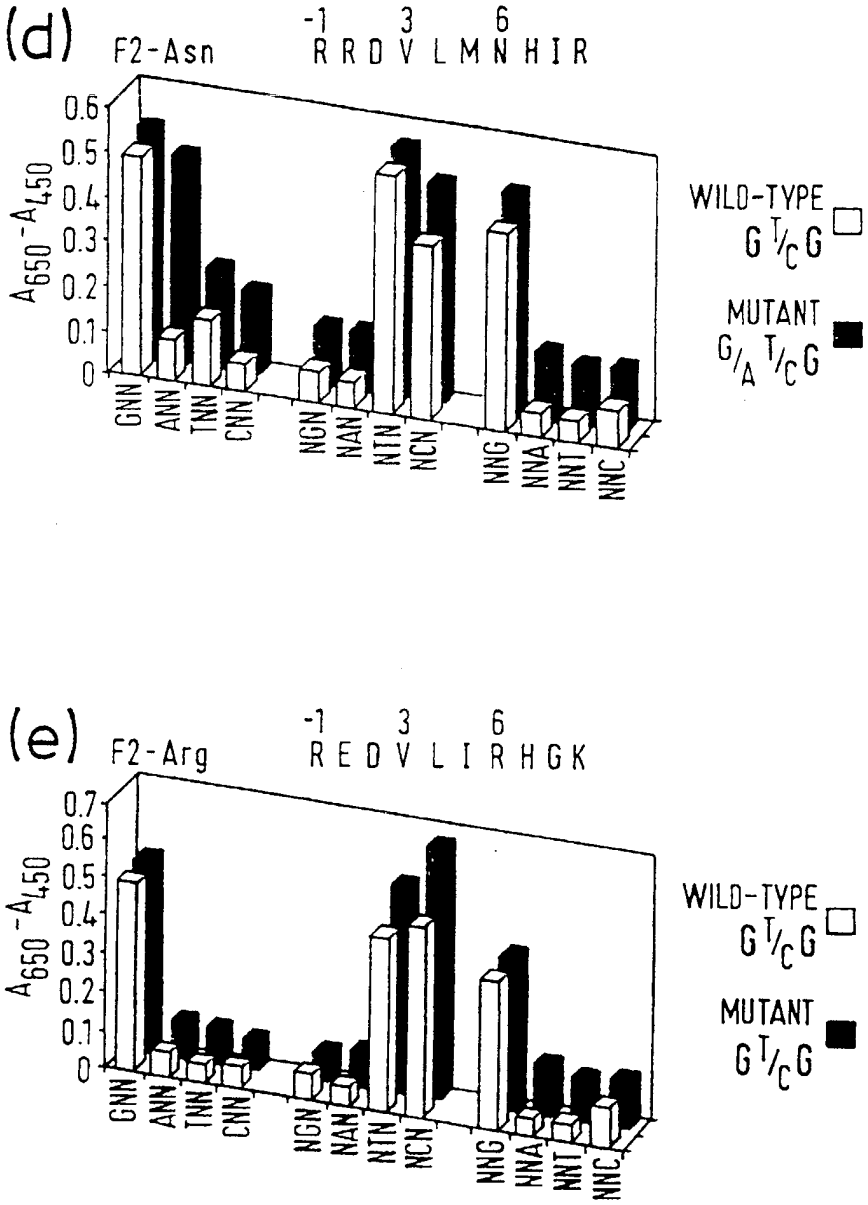
(d)
F2-Asn
-1 3 6
R R D V L M N H I R
A_{650} - A_{450}
0.6
0.5
0.4
0.3
0.2
0.1
0
GNN
ANN
TNN
CNN
NGN
NAN
NTN
NCN
NNG
NNA
NNT
NNC
WILD-TYPE
G T/C G
MUTANT
G/A T/C G
(e)
F2-Arg
-1 3 6
R E D V L I R H G K
A_{650} - A_{450}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
GNN
ANN
TNN
CNN
NGN
NAN
NTN
NCN
NNG
NNA
NNT
NNC
WILD-TYPE
G T/C G
MUTANT
G T/C G

FIG. 6 CONT'D

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 98/01516

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12N15/12 C12N15/62 C12Q1/68 C07K14/47
A61K48/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	M. ISALAN ET AL: "Synergy between adjacent zinc fingers in sequence-specific DNA recognistion" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 94, 27 May 1997, pages 5617-5621, XP002075337 WASHINGTON US see the whole document ---	1-29
A	WO 96 06166 A (MEDICAL RES COUNCIL ;CHOO YEN (SG); KLUG AARON (GB); GARCIA ISIDRO) 29 February 1996 cited in the application see the whole document see table 2 see page 33, last paragraph see page 48, paragraph III ---	1-29

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 September 1998	30/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/GB 98/01516

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9606166 A	29-02-1996	AU 3229195 A	14-03-1996
		CA 2196419 A	29-02-1996
		EP 0781331 A	02-07-1997
		JP 10504461 T	06-05-1998